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IMMUNITY IN MICE TO DRUG-ATTENUATED TRYPANOSOMA BRUCEI,(U)
MAR 79 D G MYER , A C ZAHALSKY DAMD17-74-C-4140

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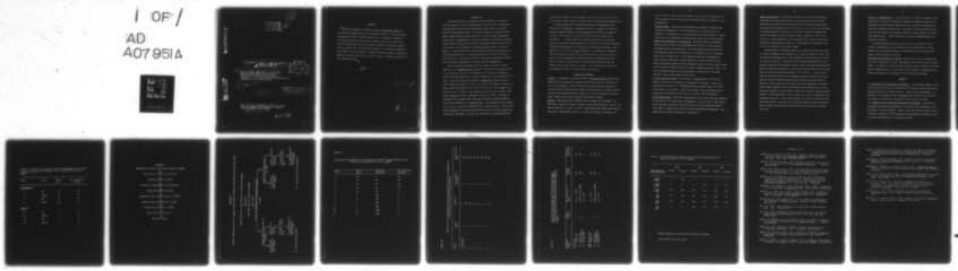

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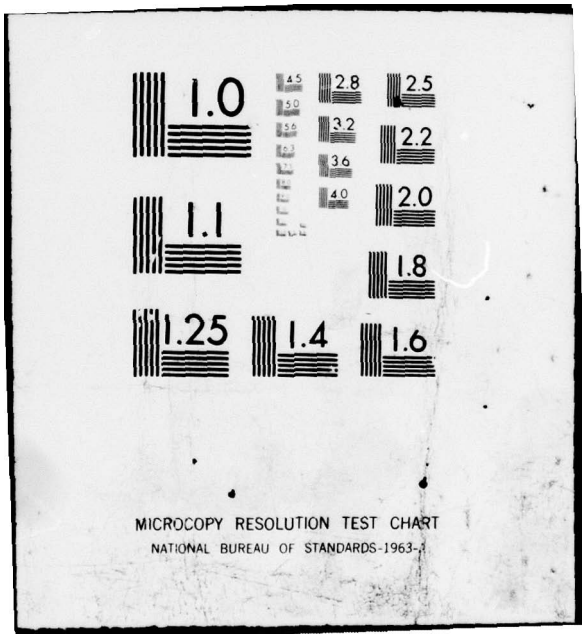
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LEVEL II

IMMUNITY IN MICE TO DRUG-ATTENUATED TRYPANOSOMA BRUCEI

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ABSTRACT

Purified, monomorphic bloodstream forms of Trypanosoma brucei were attenuated by in vitro exposure to diminazene (Berenil). Such drug-treated organisms were used to actively immunize mice. The humoral component to the observed protective immunity was examined by mouse protection tests using fractionated sera and by the passive transfer of peritoneal exudate cells or spleen cells. The protective immunity of the humoral component is attributable to both IgM and IgG. Immunity following transfer of peritoneal exudate- or spleen cells is due, at least in part, to release of antibody by the transferred cells.

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INTRODUCTION

Although doubt has been expressed on the feasibility of producing vaccine(s) against African trypanosomiases, and their value in relation to trypanosomiasis control has been questioned (Gray, 1976), the need for effective immunization remains. The vexing problems of antigenic heterogeneity among different species and strains of circulating trypanosomes, seeming lack of immunogenicity by internal antigens, and antigenic variation within species associated with surface coat glycoproteins have been cited as barriers to the development of immunoprophylaxis methods in man and animals. An approach which has been successful against some bacterial and viral diseases makes use of attenuated organisms to elicit an effective immune response. Attempts have likewise been made to attenuate salivarian and stercorarian trypanosomes to achieve effective immunization.

Live, attenuated trypanosomes have been shown to induce an active immunity, e.g., Trypanosoma cruzi exposed to Actinomycin D in vitro induced an active immunity in mice, as determined by the protection afforded to animals later challenged with a virulent homologous strain (Fernandes et al, 1965). Injection of gamma irradiated T. rhodesiense (Welcome strain) in blood conferred an immunity in mice, rats, cattle, and monkeys (Duxbury and Sadun, 1969, 1970; Duxbury et al, 1972 ; Wellde et al, 1973; Campbell and Phillips, 1976). Likewise, some immunity in rats was obtained when irradiated T. brucei were used as immunogen (James et al, 1973). Findings by Zahalsky (1974) indicated that diminazene (Berenil) reversibly inhibits nucleic acid synthesis in T. brucei in vivo. The bloodstream forms appeared not to divide or increase in numbers before clearing occurred. These observations suggested that bloodstream organisms exposed to drug in vitro might retain their immunogenic properties as attenuated, non-reproducing forms.

Trypanosomes exposed to drugs in vitro have been used to achieve an active host immunity (James, 1976), a procedure which is confirmed and extended here.

In the present study, we examine the effects of diminazene on the infectivity of monomorphic, bloodstream forms of T. brucei, and find that purified organisms exposed to drug in vitro evoke a protective immunity in mice. Some aspects of the nature of this protective immunity are determined. We also examine the relative contribution of the major classes of protective immunoglobulins in the primary and secondary responses following intraperitoneal (ip) injection of drug-exposed organisms. Advantages to the use of this in vitro system are: (i) exposure to uniformly high concentrations of drug for a controlled period of time and, (ii) the amount of drug accompanying an immunizing dose of trypanosomes (approximately 10^6 cells) is calculated to be approximately 100X less than the minimum curative dose (med) for a mouse. At this reduced level, there is no prophylactic effect of the drug in the test animals.

MATERIALS AND METHODS

Animals: A monomorphic laboratory strain of Trypanosoma brucei derived from EATRO 691A was maintained by syringe passage in mice and rats and preserved as a frozen stabilate. Unless otherwise indicated young male albino mice (outbred strain of Webster Swiss origin) and male albino rats (Wistar origin) obtained from National Laboratory Animal Co., O'Fallon, Missouri, were used. Other animals used were also obtained from the same company.

Buffers: TRIS-glucose EDTA (TG-EDTA) buffer contained the following: 5g TRIS-base, 2g glucose, 4g NaCl, 0.2g KCl, 5g Na_2 EDTA, and H_2O to 1 liter. The pH was adjusted to 7.4 with 0.1 N HCl. TRIS-glucose (TG) buffer contained the following: 5g TRIS-base, 2g glucose, 4g NaCl, 0.2g $MgCl_2$, 0.2g $CaCl_2$, and

H_2O to 1 liter. Saline buffer contained 1 M NaCl and 0.02 M TRIS-HCl; the pH was adjusted to 8.0 and sodium azide (0.001%) added to prevent bacterial contamination.

Routine Passage: Infections were maintained by passage of diluted blood containing approximately 1×10^6 trypanosomes (in a volume of 0.2 ml, TG-EDTA) by ip injection into naive 20-30g mice at 2 day intervals. Parasitemia levels were determined by hemocytometer counts of tail blood samples.

Harvest and Purification of Trypanosomes: Blood was removed from heavily infected rats (3×10^8 or more trypanosomes/ml blood) by cardiac puncture utilizing heparin as anticoagulant. The trypanosomes were concentrated by centrifuging the whole blood at 600 X g for 10 minutes (4 C) in a swinging bucket rotor (Sorvall, HB-4). The trypanosome layer was resuspended in buffer by gentle agitation, removed, washed 2X in cold TG buffer and the washed trypanosomes separated from contaminating blood cells by passage through a DEAE cellulose column eluted with TG buffer (Lanham and Godfrey, 1970). The final trypanosome suspension was hemocytometer counted and the organisms adjusted to the desired number/ml with buffer.

In Vitro Exposure to Drug: 100 µg Berenil (approximately 2×10^{-6} M) was added to each ml of cell suspension containing 10^8 organisms. Solutions of antipyrene-free Berenil (4,4'-Diamidinodiazobenzene diaceturate) were always freshly made up in TG buffer. After 30 min exposure at room temperature the suspension was diluted with buffer so as to yield 5×10^6 organisms/ml.

Active Immunization: Naive mice (approximately 20g, 6 weeks old) were immunized by injection of 10^6 drug-exposed trypanosomes ip. and rested for 7 days before being challenged, boosted or used as a source of serum, peritoneal exudate cells (PEC), or spleen cells (SC) for passive immunization experiments. This immunization procedure is summarized in Protocol I.

Passive Immunization: The humoral contribution to a protective immunity was examined by the passive transfer of pooled sera (from six actively immunized mice) diluted with normal mouse serum in order to provide varying quantities of immune sera (100, 50, 25, 12, 6, 3, 1.5 µl) per injection. A challenge dose was injected ip. 1-2 hr after injection of immune sera. Sera were obtained by cardiac puncture, pooled, allowed to clot for one half hour at room temperature, then refrigerated for 2 hours and the sera obtained by centrifuging at 2,000 × g. Serum samples were pipetted into 1 ml vials and stored at -20°C until needed.

Contributions of peritoneal exudate cells (PEC) and spleen cells (SC) to protective immunity were examined by the passive transfer of these cells from actively immunized mice to näive isogeneic mice according to the protocol shown in Protocol 2. PEC and SC were obtained from Balb/C mice which had been immunized and challenged 7 months earlier and then boosted with 10^6 Berenil attenuated trypanosomes 7 days before the cells were to be harvested. Each recipient was given approximately 5.6-7 × 10^6 PEC. After 14 days some of the PEC recipients were challenged (Group P1) whereas others were bled for serum to determine whether passive humoral protection would be afforded to other näive mice (Group P2) which were immediately challenged. SC were always obtained from the same immunized and boosted mice as those from which the PEC had been removed. Spleen cell recipients received approximately 2 × 10^8 cells/animal. After 14 days some of the SC recipients were challenged (Group S1). Other animals served as a source of serum which was then transferred to Group S2 mice to determine passive humoral protection. Control animals received cells obtained in the same manner from 10 näive donor mice.

Classes of Immunoglobulins: The contribution of the major classes of immunoglobulins to protective immunity was examined by mouse protection tests using whole sera from animals immunized once or boosted, and IgM and IgG fractions obtained from these sera. Sera mixed 2:1 with 40% sucrose solution were fractionated on a Sephadex G-200 gel column (Pharmacia K 16/100) using saline buffer. Pooled fractions of the major immunoglobulins were desalted by dialysis.

The mouse protection test was carried out by ip injection of 10^3 virulent trypanosomes (0.1 ml) mixed with one of the dilutions of whole immune sera or equivalent amounts of fractionated sera to determine the highest dilution protective to mice.

Challenges and Monitoring of Effects: Except where noted a challenge dose of 0.2 ml contained 10^3 virulent trypanosomes in blood diluted with TG-EDTA buffer. Wet smears of tail blood were routinely used to check for infections before challenge and at 3 day intervals after challenge. Experimental animals were monitored for a minimum of 30 days following challenge.

RESULTS

(i) Infectivity of drug-treated trypanosomes. A parasitemia leading to death did not occur when mice were injected with 10^6 Berenil exposed trypanosomes. Following injection of larger numbers of drug-treated trypanosomes, up to 10^8/mouse, it was observed that trypanosomes were present in wet smears of tail blood at 70 hours post innoculation but not after 114 hours.

(ii) Immunity in mice receiving drug-treated trypanosomes. Recipients of in vitro drug-treated trypanosomes were tested for their resistance to the homologous, virulent organisms. Table I shows that all forty (10 of each of 4 strains) recipients of 10^6 drug-exposed trypanosomes were immune to infection when challenged with 10^3 virulent organisms.

The requirement for live, attenuated trypanosomes in the development of a protective immunity is indicated by results obtained with drug-treated, homogenized trypanosomes and drug-treated, heat-killed organisms (58°C for 30 min). Neither of these methods resulted in an immunity when sensitized mice were challenged subsequently. Mice innoculated by the standard procedure and then challenged with varying numbers of virulent T. brucei (Table II) generally exhibit immunity when as many as 4×10^5 trypanosomes constitute the challenge dose.

(iii) Humoral contributions to protective immunity. A humoral contribution to the observed protective immunity was examined by the passive transfer of immune sera to näive mice which were challenged one hour later. Only those mice receiving less than 3 µl of immune serum became infected (Table III).

The contribution of PEC and SC to protective immunity was examined by the passive transfer of these cells from sensitized mice to näive isogeneic animals. The results in Table IV indicate that protective immunity is transferred by either PEC or SC obtained from immunized animals. That such immunity may be attributed to the release of antibody by these transferred cells can be inferred from the results obtained with Groups P2 and S2 (Table IV) which also survived challenge after receiving serum from cell recipients.

Mouse protection tests using whole sera from animals immunized once or boosted, and IgM and IgG fractions obtained from these sera indicate (Table V) protective immunity by IgM after primary immunization with increased protection after boosting attributable to an increase in both IgM and IgG.

DISCUSSION

Infectivity and Prophylactic Effect

Infection with African trypanosomes followed by drug cure to induce immunity has not been advocated as a practical means of immunization because of the danger of development of drug resistance (Gray, 1967). In this study trypanosomes exposed for 30 min *in vitro* to relatively high concentrations of drug (100 µg diminazene/10^8 trypanosomes) are non-infective when as many as 10^8 trypanosomes are injected ip. These results demonstrate the safety of this procedure compared to infection and cure where trypanosomes are exposed to relatively low drug concentrations and may be sequestered in compartments of the body not available to drug.

Girgis-Takla and James (1974) demonstrated that exposure to 5 µg diminazene (the lowest concentration employed) *in vitro* abolished infectivity of 21×10^6 *T. brucei*. James (1976) reported 10 µg diminazene as the minimum concentration required to abolish infectivity of 32×10^6 *T. brucei*. Though a curative dose of diminazene may exert a prophylactic effect in test animals even four weeks after administration (Zahalsky and Weinberg, 1976), such a prophylactic effect is absent in our system where the amount of drug (1 µg) accompanying injection of 10^6 trypanosomes is near 100 times less than a curative or prophylactic dose for a 20 g animal.

Resistance of Actively Immunized Mice to Challenge

10^6 drug-treated trypanosomes induce a solid protective immunity in mice against challenge 1 week later with 5×10^4 virulent, homologous organisms. In a majority of mice challenged with 4×10^5 organisms protection is obtained. These results are comparable to the findings of James (1976). Earlier, Duxbury & Sadun (1969) found that 83% of mice inoculated with 1 dose of 2×10^6 irradiated *T. rhodesiense* survived a challenge 1 week

later of 10^3 homologous parasites, whereas 100% survival was observed in mice which had received 2 or 3 immunizing inoculations. Duxbury and Sadun (1970) further found that 60% of mice inoculated with 3 doses of 4×10^6 irradiated T. brucei survived a challenge of 10^3 homologous parasites, while none survived challenge with T. gambiense or T. rhodesiense. Irradiated T. gambiense and T. rhodesiense conferred complete protection against homologous challenge, but no protection against heterologous challenge. Rats immunized with 2×10^6 radioattenuated T. brucei showed 74 and 33% survival when challenged 10-20 days later with approximately 1×10^4 or 2×10^5 homologous parasites, respectively (Fregene et al, 1975). When these results are compared to the findings obtained here, it is to be noted that the immunity conferred by these chemically attenuated forms is greater than that elicited by irradiated trypanosomes.

Passive Immunity

Our studies show that as little as 3 µl of immune serum protects näive mice against a challenge of 1×10^3 homologous trypanosomes. If we assume that the blood volume of a 25g mouse is 2 ml, with half this amount being serum, then the protection afforded an actively immunized mouse against challenge with 4×10^5 virulent trypanosomes appears to be entirely humoral in nature. Our experiments also indicate that the protection obtained in mice by the passive transfer of PEC and SC is due, at least in part, to humoral contributions. Takayanagi et al (1973) found that spleen cells obtained from mice 1 to 10 days after a single immunization against T. gambiense protected recipient näive mice against challenge with homologous trypanosomes five hours later. In these studies a minimum of 3×10^7 immune spleen cells from donor mice on day 5 after immunizations were required to protect recipient mice against a challenge of 1×10^4 organisms, with twenty-three times as many spleen cells required if taken 30 days

after immunization. Similarly, Campbell and Phillips (1976) using irradiated T. rhodesiense to immunize mice found significant adoptive transfer of protection with immune serum, unfractionated immune spleen cells, and immune B-enriched spleen cells. A comparison of these findings with the results obtained here is difficult to make since the parasite, host strain, time of challenge and number of parasites in the challenge differed. Although the data of both Takayanagi et al and Campbell and Phillips suggest that immune spleen cells elicit the appearance of serum factor(s) (antibody-mediated mechanisms), specific evidence of humoral protection in experimental animals receiving immune spleen cells was not presented.

Although the results reported here using passively transferred SC or PEC in the mouse system give emphasis to a humoral contribution to protective immunity, the issue of a cell-mediated immunity has been addressed by others. Tizard and Soltys (1971) found an immediate (humoral mediated) and a delayed (cell-mediated) hypersensitivity in rabbits infected with T. brucei when these animals were skin-tested with homologous antigen. In this case the delayed hypersensitivity was shown to be transferrable with certain strains using live SC but not dead SC or serum. Presumably, an immediate hypersensitivity could have been demonstrated by them with either live spleen cells or serum. The aforementioned results are not inconsistent with those of Campbell and Phillips (1976) who found no adoptive transfer of protection with T-cell enriched immune spleen cells. This non-adoptive transfer of protection neither excludes a T-cell contribution in helper or memory mode nor a delayed hypersesntivity response, which in their system conferred no protection. However, Pouliot et al (1977) reported that resolution of a T. musculi infection in mice is not attributable to a humoral response, appears to be thymus dependent, but does occur in T-cell deprived mice receiving immune or normal spleen cells

even when these cells are treated with anti-θ serum. The absence of a humoral response in a resolving T. musculi infection is perplexing, as is a resolution of infection when T-cells are removed by anti-θ treatment.

Our results with fractionated sera obtained after primary immunization indicate that the protection conferred is virtually fully attributable to IgM and that subsequent to the anamnestic response protection is conferred to a greater extent by IgM although the overall response results in increased levels of both classes of immunoglobulin. These findings are consistent with those of Takayanagi and Enriquez (1973) who demonstrated that the IgM fraction of immune serum from a primary immunization against T. gambiense was effective at a much lower concentration than IgG in protecting mice against challenge with homologous organisms. Our results on protection using fractionated sera, when examined in conjunction with the results on agglutination obtained by Seed et al (1969) using T. gambiense and by Weinberg and Zahalsky (1976) using T. brucei, support the suggestion of Takayanagi and Enriquez (1973) that there is little parallel relationship between the agglutination titer and protective ability in the course of immunization.

ACKNOWLEDGEMENTS

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Table I. Survival of mice immunized with 10^6 Trypanosoma brucei exposed to Berenil in vitro and challenged 7 days later with 10^3 virulent T. brucei

Group	Strain	No. of mice	No. surviving 30 days
Experimentals			
1	NL	10	10
2	BALB/C	10	10
3	AKR	10	10
4	C57BL.	10	10
Controls			
1a	NL	2	0
2a	BALB/C	2	0
3a	AKR	1	0
4a	C57/BL.	1	0

Protocol 1

Immunization procedure with drug-attenuated *T. brucei*

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Infect rats (5×10^6 to 1×10^7 ip.)

↓

Harvest by cardiac puncture

↓

Concentrate trypanosomes by centrifugation

↓

Purify through DEAE cellulose

↓

Resuspend in Tris-glucose buffer (1×10^8/ml)

↓

Expose to 100 µg Berenil/ml for 30 min

↓

Dilute with buffer to 5×10^6/ml

↓

Inject mice with 0.2 ml ip.

↓

Rest mice 7 days

Protocol 2

Passive immunity in mice to T. brucei using Peritoneal Exudate Cells (PEC) and Spleen Cells (SC).

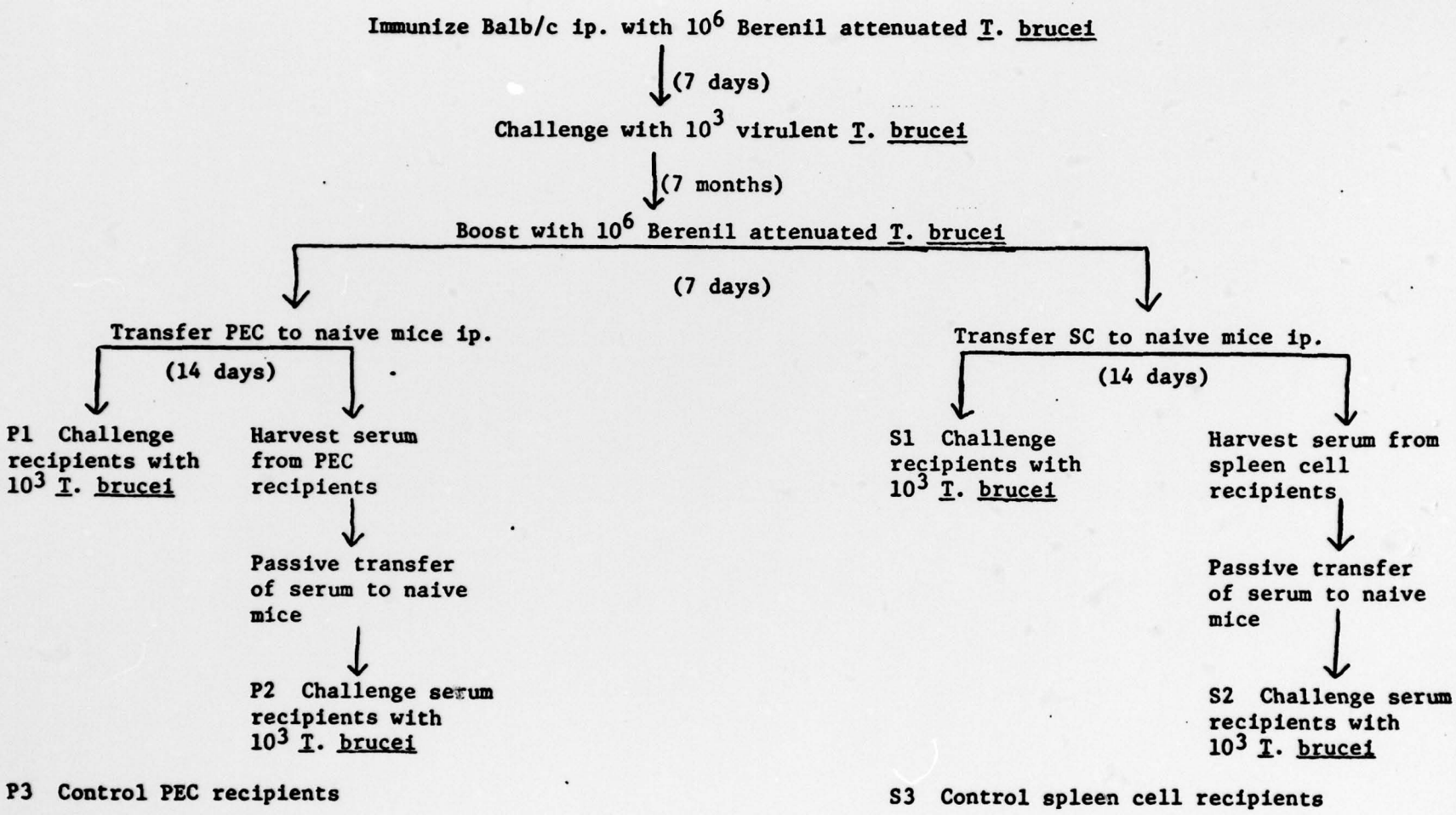

Table II

Survival of mice immunized 7 days previously with 10^6 T. brucei exposed to drug in vitro and challenged later with T. brucei

Group	No. of mice	Challenge dose x 10^3	No. surviving >30 days
1	10	1	10
2	10	30	10
3	10	40	10
4	10	50	10
5	10	60	9
6	3	100	3
7	3	200	2
8	3	400	2
9	3	800	0
10	3	1200	0
11	3	1600	0

Table III.

Survival of mice challenged with 10^3 T. brucei following passive immunization
(sera from mice immunized 7 days previously)

Group	No. of mice	Serum, µl	No. surviving challenge	Survival time, days
1 (Control)	3	0	0	5-6
2	3	100	3	>30
3	3	50	3	>30
4	3	25	3	>30
5	3	12	3	>30
6	3	6	3	>30
7	3	3	3	>30
8	3	1.5	0	5-9

Table IV. Mice surviving challenge with 10^3 infective T. brucei following passive immunization with Peritoneal Exudate Cells or Spleen Cells obtained from previously immunized animals.

Recipient groups	Number of mice	No. of PEC or SC given	Percent survival	Survival time, days
PEC Groups				
P1 Immune PEC	2	$5.6 - 7 \times 10^6$	100	>30
P2 Serum from PEC recipients	3	NONE (0.1 ml serum)	100	>30
P3 Control PEC	5	6.5×10^6	0	5
Spleen Cell groups				
S1 Immune SC	2	2.1×10^8	100	>30
S2 Serum from SC recipients	3	NONE (0.1 ml serum)	100	>30
S3 Control SC	5	2.3×10^8	0	5-6

Table V. Protective immunity afforded mice by whole and fractionated sera against challenge with 10^3 T. brucei*

	Whole		IgM		IgG	
Serum Dilution / Amt. serum/mouse	Primary	Secondary	Primary	Secondary	Primary	Secondary
5,000 / 0.2µℓ	---	0/3	---	---	---	---
2,000 / 0.5µℓ	---	---	---	0/3	---	0/3
1,000 / 1µℓ	1/3	3/3	0/3	1/3	0/3	0/3
200 / 5µℓ	2/3	3/3	2/3	3/3	0/3	1/3
100 / 10µℓ	3/3	3/3	3/3	3/3	1/3	3/3
40 / 25µℓ	---	---	3/3	3/3	0/3	---

*figures indicate no. surviving >30 days/no. challenged .

All controls died in 5-6 days.

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